Transfection Polymers

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Solid-Phase Synthesis of Sequence-Defined T-, i-, and U-Shape Polymers for pDNA and siRNA Delivery**

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Viral proteins are far more effective in mediating the transport of viral nucleic acids into cells than the currently available synthetic polymers for gene transfer. To mimic viral delivery processes, functional domains such as endosomolytic agents and targeting ligands have been conjugated to polymers. The chemistry of such conjugates, however, lacks the molecular precision of sequence-defined viral proteins, regarding both the polydispersity of the polymer and the conjugation sites. The common practice to apply such polydisperse mixtures in transfections may obscure accurate structure–activity relationships. It is questionable whether polydisperse macromolecules will ever compete successfully with their viral counterparts. Herein we communicate on the solid-phase-supported synthesis of a small library of sequence-defined polymers and their use for pDNA and siRNA delivery.

Solid-phase-supported macromolecule assembly[1] has already been applied for nucleic acid carriers.[2,3] Hartmann, Börner, and colleagues published solid-phase-based syntheses of polyamidoamines employing alternating condensation steps using succinic anhydride and diamino-*N*-methyldipropylamine or protected spermine.[4] To combine the advantages of peptide synthesis with the broader chemical diversity of synthetic polymers, we designed artificial Fmoc/Boc-protected amino acids with defined diaminoethane units.[5] The protonatable diaminoethane motif has unique properties as a "proton sponge" for the endosome buffering and destabilization responsible for the high transfection activity of polyethylenimines (PEI)[6,7] and other polymers.[8] The biological activity of diaminoethane units is far superior to that of diaminopropane units, which are completely protonated at physiological pH.[7d,8a]

The three artificial amino acids (Stp, Gtp, and Gtt; Figure 1a) were applied together with lysines (as branching units), cysteines (bioreversible disulfide-forming units), and various fatty acids (stabilizing hydrophobic domains) to generate a small library of more than 300 defined structures. As different topologies can influence the complexation and biological properties of transfection agents,[6c,9] linear polycations with or without modification in the center (T-shapes) or the end of chains (i-shapes, U-shapes) as well as branched structures were explored (Figure 1b).

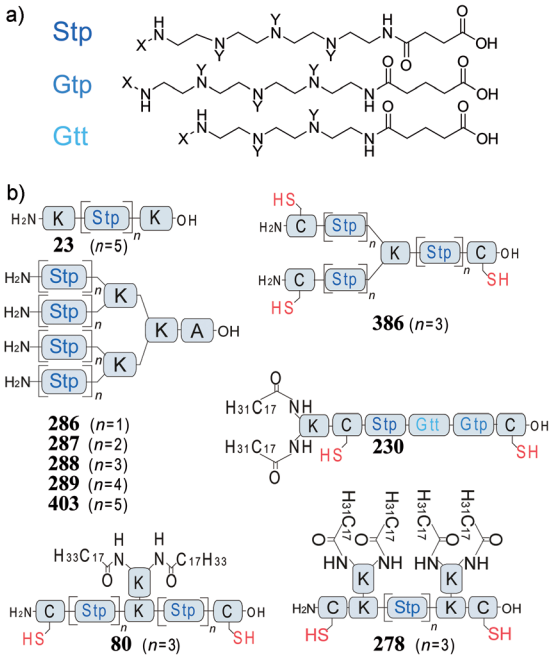

Figure 1. a) Oligo(ethylene amino) acids without (X = Y = H) or with protective groups (X = Fmoc, Y = Boc). b) Polymers. Linear chains (**23**), four-armed structures (**286**–**289** and **403**), chains with diacylation at the center (T-shape) and cross-linking cysteines (**80**); three-armed structure with three cross-linking cysteines (**386**); chains with diacylation at the N terminus (i-shape) and two cross-linking cysteines (**230**), chains with two diacylation sites and cross-linking cysteines (U-shape, **278**). K = lysine, C = cysteine.

Figure 2 presents the luciferase gene transfer activity of selected polymers complexed with pDNA at indicated protonatable nitrogen/phosphate (N/P) molar ratios. Polymers

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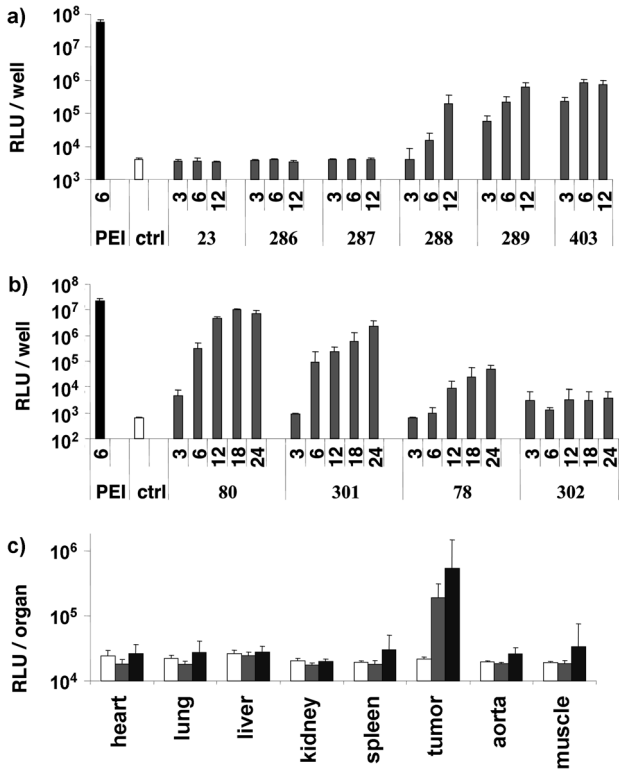

Figure 2. Transfection of Neuro2A cells with pDNA polyplexes. a) Four-armed structures. b) T-shape **80** and its mutants. LPEI used as positive control. c) Luciferase gene expression in vivo after systemic polyplex administration in tumor-bearing mice. White: **78**, gray: **80**, both w/w 20; black: G3-HD-OEI[7d] as positive in vivo control.

Table 1: Selected polymer sequences with analogues.

Polymer	Sequences [from N to C terminus][a]
80	C-Stp-Stp-Stp-$(OleA)_2$K]K-Stp-Stp-Stp-C
301	A-Stp-Stp-Stp-$(OleA)_2$K]K-Stp-Stp-Stp-A
78	C-Stp-Stp-Stp-K] K-Stp-Stp-Stp-C
302	A-Stp-Stp-Stp-K]K-Stp-Stp-Stp-A
386	$(C\text{-Stp-Stp-Stp})_2$]K-Stp-Stp-Stp-C
387	$(A\text{-Stp-Stp-Stp})_2$]K-Stp-Stp-Stp-A
230	$(LinA)_2$K-C-Stp-Gtt-Gtp-C
377	K-C-Stp-Gtt-Gtp-C
378	K-Stp-Gtt-Gtp-A
379	$(LinA)_2$K-Stp-Gtt-Gtp-A
278	C-$(LinA)_2$K]K-Stp-Stp-Stp-$(LinA)_2$K]K-C
279	A-$(LinA)_2$K]K-Stp-Stp-Stp-$(LinA)_2$K]K-A

[a] "]K" represents a lysine with branching at the α,ε-amino groups.

like the linear chain **23** or branched structures **286** and **287** with less than 10 Stp units (i.e. < 50 nitrogen atoms) do not transfect, consistent with their low DNA-binding ability (Figure S1 A in the Supporting Information) and the properties of 800 Da oligoethylenimine.[7d] Branched polymers **288**, **289**, and **403** (with 60, 80, and 100 Stp nitrogen atoms, respectively) effectively bind DNA through electrostatic interactions, as shown by agarose gel retardation assays (Figure S1 A) and form 80–100 nm polyplexes (Table S1 in the Supporting Information). They show gene-transfer activity (Figure 2 a) about 100-fold above the background of untransfected cells (ctrl), but still 100-fold lower than the gold standard, linear PEI with 22 kDa.

Four-armed polymers were generated to obtain cationic branched structures of high molecular weight in few synthetic steps. Modification of smaller polymers with a hydrophobic domain and two cysteines (**80**) strongly improved DNA delivery (Figure 2 b). Cysteine-containing polymers **80** and analogue **78** (Table 1) show accelerated disulfide-bond formation in the presence of DNA (Figure S3) and far better DNA binding than the alanine analogues **301** and **302** (Figure S1 in the Supporting Information). Hydrophobic modification by the incorporation of α,ε-diacylated lysine at the center of the polymer chain (T-shape) enhanced polyplex stability (Figure S1 in the Supporting Information). When we screened mono- and diacylation with fatty acids 4–20 carbons in length, we identified lysine diacylation with 14–18 carbon-chain fatty acids as the most effective. Polymer **80** containing cysteines and two oleoyl units mediates high transfection (Figure 2 b), far higher (at N/P 12: 30-, 500-, > 1000-fold) than analogues lacking cysteine (**301**), oleic acid (**78**), or both (**302**). Cytotoxicity was not observed with any transfection (Figure S4 in the Supporting Information).

Based on the encouraging performance, in vivo studies were performed in A/J mice bearing subcutaneously growing Neuro2A neuroblastoma. Polyplexes formed with **80** and pDNA at a concentration of 200 μg mL^{-1} were stable in serum and had a uniform size around 155 nm (Figure S2 and Table S1 in the Supporting Information). Intravenous application resulted in gene expression in tumors but not any other organ (Figure 2 c), which is consistent with the expression profile of the positive control, polymer G3-HD-OEI.[7d] In contrast, analogue **78** lacking oleic acid did not mediate detectable transfection.

DNA stability can be ruled out as the only reason for the positive effect of diacylation with oleic acid (**80** versus **78**) on cysteine-stabilized polyplexes. In fact, the less stable polyplex formulation of cysteine-free polymer **301** induced higher gene transfer than **78** (Figure 2 b). The hydrophobic group introduces an additional advantage. After cell entry by endocytosis, an endosomal pH-dependent lytic activity is beneficial in facilitating the escape of polyplexes from endosomes to the cytosol. Thus, polymers were screened in erythrocyte leakage assays[10] to identify motifs resulting in pH-dependent lysis (Figure 3). Preferred activity at lower pH could prevent toxic lysis of cell membranes at physiological pH.

The rather hydrophilic polymers lacking hydrophobic modifications are inactive in the lysis assay. Polymers diacylated with oleic acid (**80**, **301**) or linolic acid (**278**, **230**, **379**) showed a pH-dependent lysis most pronounced at pH 5.5. pH-responsive protonation of oligoamines increases the cationic character for membrane binding, the diacyl chains provide amphipathic character. Both, consistent with literature,[3b] appear to be required for lytic activity. Diacylation with myristic acid triggers the highest but less pH-specific lytic activity, resulting in cytotoxic polymers. Because of the pH-dependence of lysis, polymers **80**, **301**, and **278** displayed no detectable cytotoxicity up to the highest tested concentration (100 μg mL^{-1}, see Figure S5). Only linolic acid

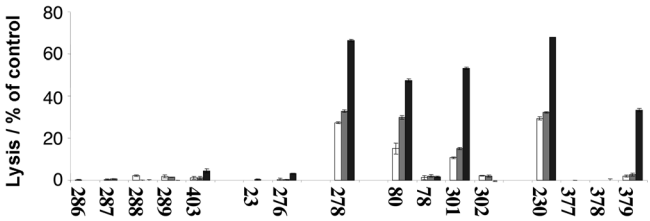

Figure 3. Erythrocyte lysis assay at different pH values. Erythrocytes were incubated with 2.5 μM polymer solutions at 37 °C and the indicated pH. Hemoglobin release was measured after 1 h. White: pH 7.4, gray: pH 6.5, black: pH 5.5.

modified polymer **230** showed moderate cytotoxicity, which was still ten times lower than that of LPEI.

We also applied the new platform to discover siRNA carriers. Owing to the smaller size of siRNA, polyplex stabilization induced by cysteine disulfide cross-links or extended hydrophobic modification was found to be even more critical than for pDNA. All DNA carriers in Figure 2 apart from **80** were inactive in siRNA delivery. Figure 4 and Figure S6 in the Supporting Information describe examples from three polymer classes that are highly effective in siRNA transfer.

Three-armed Stp-based polymers like **386**, which contain three cysteines for cross-linking, display gene-sequence-specific silencing over a broad range of polymer/siRNA N/P ratios, as demonstrated in Neuro2A cells expressing eGFP-luciferase (Figure 4a). Replacing cysteines by alanines generates inactive **387** with very low siRNA binding (Figure S1B in the Supporting Information).

The second polymer class contains two cysteines and a hydrophobic domain at the N terminus ("i-shape"). Figure 4b shows silencing by polymer **230**, based on sequence Cys-Stp-Gtt-Gtp-Cys and a terminal di(linolic acid)-modified lysine. Deleting either the hydrophobic domain (**377**) or the cysteines (**379**) or both (**378**) results in loss of silencing, loss of siRNA binding (**377**, **378**, Figure S1B) and loss of stable particle formation (**379**, Table S2 in the Supporting Information). In cell uptake studies using Cy5 labeled siRNA (Figure S7), **230** mediates intensive siRNA uptake into all cells, **379** a moderate uptake, **377** and **378** no uptake. Altering the sequence of the artificial amino acids also may change the activity.

The third class of polymers are modified both at the C and N termini with diacylated hydrophobic domains ("U-shape"). Polymers may also contain two cysteines for stabilization. As shown in Figure 4c, both the polymer **278** (with cysteines) and its alanine analogue **279** mediate siRNA-specific silencing. Apparently hydrophobic stabilization by four linoleic acid residues can compensate the lack of covalent disulfide linkages.

In summary, we have described the use of novel, protected artificial oligo(ethylene amino) acids for the solid-phase-supported synthesis of sequence-defined polymers offering precise modification patterns and topology. Our first examples already demonstrate proof of concept for the high potential of such polymers for nucleic acid delivery. Notably, the chemistry can be used for incorporation of targeting ligands such as peptides or small molecules and shielding agents such as polyethylene glycol. These findings and a detailed analysis of the influence of polymer sequences on carrier efficacy will be reported in due course.

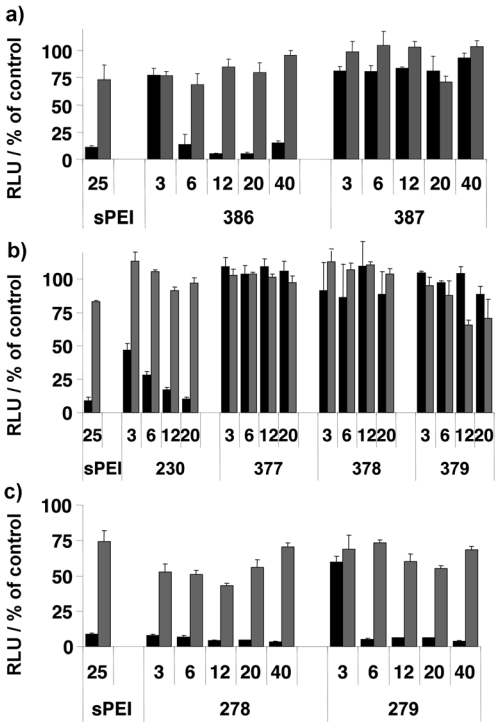

Figure 4. siRNA polyplexes (a: three-armed, b: i-shape, c: U-shape) for gene silencing in Neuro2A-eGFPLuc cells. eGFP-targeted siRNA or control siRNA was tested. Positive control: succinylated PEI (sPEI).[11] Black: GFP-siRNA, gray: control siRNA.

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